Detection of Sulfur Mustard-Induced DNA Damage by a ^{32}P-Postlabeling Method

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ABSTRACT

We have applied the ^{32}P-postlabeling technique to the detection of sulfur mustard-induced damage in white blood cell DNA. Red blood cells are lysed, and DNA is released from white cells by proteinase K treatment; proteins are removed by salt precipitation and DNA is collected by ethanol precipitation. Digestion to the 3'-deoxynucleotide level is performed with micrococcal nuclease and spleen phosphodiesterase, and the mixture of normal and modified nucleotides is labeled by T4 kinase using γ-[^{32}P]ATP. The resulting deoxynucleoside-3',5'-diphosphates are converted to deoxynucleoside-5'-phosphates with P1 nuclease. Most of the radioactivity can be removed by passage through a disposable anion exchange column because the major adduct, 7-hydroxyethylthioethyl deoxyguanosine 5'-phosphate (HETEpdG), elutes well ahead of normal deoxynucleotides and residual γ-[^{32}P]ATP. The eluent containing HETEpdG is concentrated and separated on a C-18 column by high performance liquid chromatography; one min fractions are collected and counted in a scintillation counter. The 7-hydroxyethylthioethyl guanine content in the DNA is then determined from the radioactivity associated with the HETEpdG peak. Advantages of this technique include its femtomole sensitivity and its requirement for only one microgram of DNA as well as its potential ability to detect other sulfur mustard-induced DNA adducts which may have more biological significance than 7-hydroxyethylthioethyl guanine.

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INTRODUCTION

Analytical methods that measure DNA damage should indicate the severity of cellular exposure to sulfur mustard since both the acute and chronic toxicities of this agent are apparently initiated by DNA damage (1). These methods can be used in laboratory studies to evaluate protective regimens, or potentially in a clinical situation to predict the severity of the systemic toxicity which would be expected from any actual exposure. Table 1, which is adapted from Phillips (2) and from Wogan and Gorelick (3), summarizes the capabilities of detection methods that have been used to measure other DNA adducts.

Table 1 - Analytical Methods for the Detection of DNA Adducts

Method	Detection Limit (fmol)	μg DNA required	Adduct: nucleotides
Immunological			
ELISA competitive	1	50	$1:6 \times 10^8$
ELISA non-competitive	3	0.1	$1:1 \times 10^7$
GC-MS	0.5	-	-
HPLC			
UV detection	1×10^5	1,000	$1:3 \times 10^4$
Fluorescence detection	5×10^3	1,000	$1:1 \times 10^6$
Electrochemical detection	500	100	$1:1 \times 10^6$
^{32}P-Postlabeling	0.01	1-10	$1:3 \times 10^9$

The ^{32}P-postlabeling technique, introduced by Randerath (4) and reviewed recently by Gupta (5), has been used successfully to measure DNA adduct formation by several environmental agents (2, 6-8). This method depends on the action of T4 kinase to transfer a [^{32}P]-phosphate group from γ-[^{32}P]ATP to the 5′ position of a 3′ deoxynucleotide. γ[^{32}P]ATP can be obtained with a very high specific activity which allows femtomole (10^{-15}) or even attomole (10^{-18}) quantities to be detected. Normal nucleotides are also labeled by T4 kinase, however, so that the success of the method depends on developing separation techniques which allow the adduct to be measured in the presence of much higher concentrations of normal nucleotides. Synthesis and characterization of the modified 3′- and 5′-deoxynucleotides is essential to the development of the required separations.

Analysis of 7-substituted deoxyguanylic acids similar to those formed by sulfur mustard has been problematic, however, because of the instability of these derivatives (6). Shields *et al.* (9) have developed a method which is successful for measuring the relative simple adduct, 7-methyl deoxyguanylic acid, but their techniques have not been applied to more complex derivatives. In this manuscript, we describe the successful determination of the principal sulfur mustard-induced DNA adduct, 7-hydroxyethylthioethyl guanine.

MATERIALS AND METHODS

Materials

[^{14}C]-Sulfur mustard (0.88 mCi/mmole), uniformly labeled in the chloroethyl group, was supplied by the Analytical Chemistry Branch, U.S. Army Institute of Chemical Defense. Hemisulfur mustard was synthesized by the method of Tsou *et al.* (10). γ-[^{32}P]ATP was obtained from New England Nuclear; T4 kinase, from New England BioLabs; micrococcal nuclease, ATPase, P1 nuclease, and proteinase K, from Sigma; and spleen phosphodiesterase, from Worthington. 7-Hydroxyethylthioethyl deoxyguanosine 3'-phosphate (HETEdGp) and 7-hydroxyethylthioethyl deoxyguanosine 5'-phosphate (HETEpdG) were prepared by reacting the corresponding deoxynucleotides with hemisulfur mustard in phosphate buffer, pH 3.5. Calf thymus DNA was modified with [^{14}C]-sulfur mustard (24 μM) in 50 mM sodium cacodylate buffer, pH 7, and washed free of non-covalently bound material by repeated alcohol precipitations. The product contained 630 fmol/nmol DNA total alkylation as calculated from the specific activity of the [^{14}C]-sulfur mustard. The 7-hydroxyethylthioethyl guanine content, determined by acid depurination and high performance liquid chromatography (HPLC) analysis, was 380 fmol/nmol DNA. This substrate was blended with unmodified DNA to obtain more lightly alkylated substrate for calibration of the ^{32}P-postlabeling method. Human blood, used for *in vitro* studies, was obtained from volunteers using a protocol that had been approved both by the local and Army Institutional Review Boards.

Postlabeling Techniques

The ^{32}P-postlabeling procedure was performed in discrete steps as described in the literature (5). These are numbered as follows in Figure 1 (next page): (*1*) digestion of DNA to 3'-deoxynucleotides (dNp's) with a combination of micrococcal nuclease and spleen phosphodiesterase; (*2*) immediate labeling with T4 kinase and γ-[^{32}P]ATP; (*3*) P1 nuclease treatment to remove 3' phosphates to improve chromatographic separations in the next steps; (4) removal of ATP and labeled, unmodified 5'-deoxynucleotides (pdN's); and (5) HPLC analysis.

Two steps are particularly important in our adaptation of the ^{32}P-postlabeling method to the detection of the principal sulfur mustard adduct, HETEpdG. First, since addition of the 5'-phosphate stabilizes the 3' adduct, T4 kinase labeling is performed immediately after digestion and before any separation steps. Second, since HETEpdG elutes from an anion

exchange column ahead of ATP and unmodified deoxynucleotides, HETEpdG can be separated from approximately 95% of the radioactivity on a 1 ml Sephadex A-25 column. An optical marker for HETEpdG is added to the sample in step 4 and the labeled sample is applied to an A-25 column contained in a 1 ml disposable syringe. The sample is eluted at 1 ml/min with 70 mM triethylammonium acetate buffer, pH 7, and fractions containing the optical marker for HETEpdG are collected and concentrated by lyophilization; the syringe and its remaining radioactive contents are removed from the apparatus and discarded in the radioactive waste. The lyophilized sample which contains the [^{32}P]-labeled HETEpdG and the optical marker, as well as some [^{32}P]-labeled, unmodified nucleotides is separated on a C-18 column eluted at 1 ml/min with the same 70 mM triethylammonium acetate, pH 7, buffer; the eluent is monitored at 254 nM. One min fractions are collected, and the radioactive content is determined by counting the Cherenkov radiation in a scintillation counter. Radioactivity in the peak corresponding the HETEpdG marker is calculated by a computer program which automatically subtracts background.

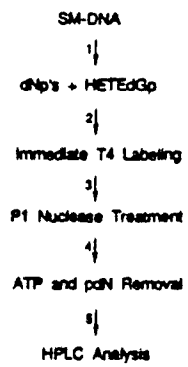

Fig. 1: Steps in the detection of SM damage by the ^{32}P-postlabeling technique. See text for description of each step.

Isolation of DNA from SM-treated Blood

To demonstrate the application of the ^{32}P-postlabeling method to a biological sample, 20 ml of human blood was anticoagulated with 2 ml of heparin and [^{14}C]-sulfur mustard was added to a final concentration of 200 μM. Incubation was continued for 2 hrs at 37°C, and then the red blood cells were lysed and the white blood cells collected by centrifugation. DNA was released from white cells by proteinase K treatment, proteins were precipitated with ammonium acetate, and DNA was recovered by ethanol precipitation. The DNA was redissolved in water and extracted twice with a chloroform-isoamyl alcohol mixture (3/1, vol/vol) and finally reprecipitated with ethanol. Purified DNA was dissolved in water and digested as described above. A quantity corresponding to 0.33 μg (1 nmol of nucleotides) was subjected to ^{32}P-postlabeling as described above.

RESULTS

The 7-hydroxyethylthioethyl guanine content of DNA exposed to sulfur mustard should provide a reliable measure of all modifications present since the distribution of sulfur mustard-induced DNA modifications remains constant as the total alkylation is increased (10). 7-Hydroxyethylthioethyl guanine accounts for approximately 60% of total DNA alkylation by sulfur mustard making this adduct the preferred candidate for analysis (11-12).

The key enzymatic steps in the ^{32}P-postlabeling detection of 7-hydroxyethylthioethyl guanine (HETEG) are shown in Figure 2. The 3'-deoxynucleotide of HETEG, 7-hydroxyethylthioethyl deoxyguanosine 3'-phosphate (HETEdGp), is converted to the [^{32}P]-labeled 7-hydroxyethylthioethyl deoxyguanosine 3',5'-diphosphate (HETEpdGp) by T4 kinase. P1 nuclease, in step 3, removes the 3' phosphate from HETEpdGp and other nucleotides, thereby improving the chromatographic separations in steps 4 and 5.

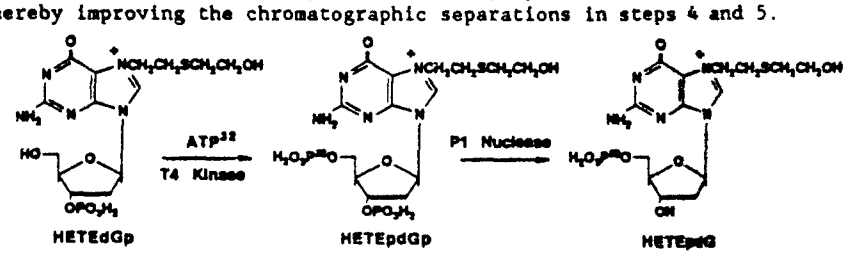

Fig. 2. Enzymatic steps in the ^{32}P-postlabeling analysis of HETEG in DNA.

A finding critical to the development of our analytical scheme is that HETEdGp is much less stable than HETEpdG. Data illustrating this point are shown in Figure 3. This difference in stability has led us away from the procedure described by Shields *et al.* (9) in which a separation is performed on the 3'-deoxynucleotides. Instead, we label with T4 kinase immediately and perform separations at the 5'-deoxynucleotide levels.

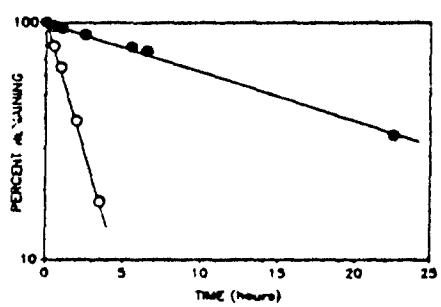

Fig. 3. Chemical stability of 7-hydroxyethylthioethyl deoxyguanosine 3'-phosphate (O) and of 7-hydroxyethylthioethyl deoxyguanosine 5'-phosphate (●) at 37°C and pH 7.

Figure 4, left panel, shows the detection of 13 fmol of HETEpdG in 0.33 μg of DNA (1 nmol of nucleotides). This quantity of sulfur mustard-modified DNA was digested and labeled as described above and separated on a C-18 column eluted at 1 ml/min with 70 mM triethylammonium acetate buffer, pH 7. Labeling of 0.33 μg of control DNA is shown in the right hand panel; it is clear that 13 fmol of HETEpdG is easily detected above background. This experiment was performed with [^{32}P]ATP which had a specific activity of 40 Ci/mmol; greater sensitivity can be achieved by increasing this figure. Further increases in sensitivity can probably be achieved by a treatment with P1 nuclease prior to the labeling step (13).

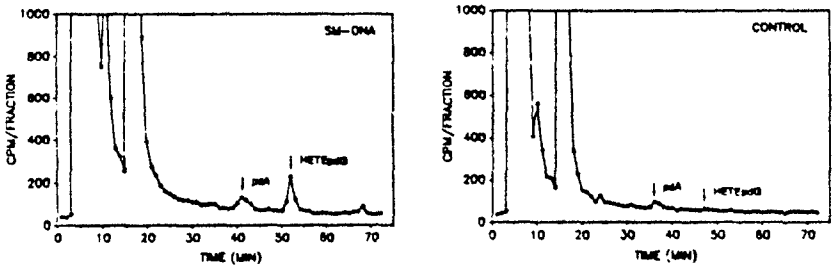

Fig. 4. Left panel: Detection of 13 fmol of HETEpdG in 0.33 μg of sulfur mustard-treated DNA. Right panel: Analysis of 0.33 μg of control DNA.

Figure 5 shows the calibration curve obtained by analyzing blends of DNA containing from 6 to 380 fmol of HETEG. In these analyses, 0.33 μg of DNA (equal to 1 nmol of nucleotides) was analyzed; therefore, alternate units for the x axis are number of HETEG adducts/10^6 nucleotides. Increased precision can be achieved by including an internal standard, but it is clear from this Figure that subfemtomole detection will be possible by increasing the specific activity of the [^{32}P]ATP and/or by P1 nuclease treatment prior to labeling.

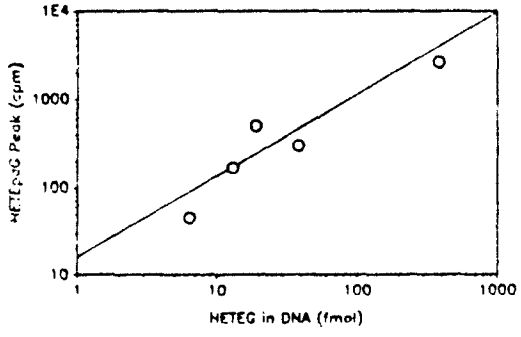

Fig. 5. Calibration of the ^{32}P-postlabeling method. 0.33 μg of DNA (1 nmol) containing varying amounts of HETEG was analyzed as described in the text and the size of the HETEpdG peak was plotted versus the amount of HETEG in the DNA.

Figure 6 shows the application of this method to the detection of HETEpdG in white blood cell DNA recovered from human blood exposed *in vitro* to 200 μM of [^{14}C]-sulfur mustard. Only 0.33 μg of DNA was used in this analysis so that it is clearly applicable to situations where only limited amounts of DNA are available. (0.33 μg is approximately the amount of DNA which can be isolated from 10^6 cells.) Using the calibration curve shown in Figure 5, the level of modification in this DNA is 22 fmol/nmol of DNA nucleotides.

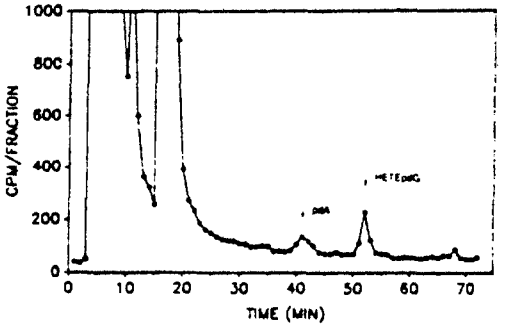

Fig. 6. Detection of HETEpdG in DNA extracted from whole blood exposed to 200 μM sulfur mustard.

DISCUSSION

The results shown above establish ^{32}P-postlabeling as a method which can easily detect one HETEG in 10^5 nucleotides. The sensitivity of the method allows the determination to be made on very small amounts of DNA. As markers are synthesized for other sulfur mustard-induced DNA adducts and methods of separation are developed, this method will provide information on other sulfur mustard-induced modifications of interest.

Two modifications of the standard ^{32}P-postlabeling technique have been important in making its application to the detection of HETEG possible. First, immediate T4 kinase labeling of the HETEdGp released by enzymatic treatment stabilizes this adduct and results in high labeling efficiency. Second, the use of a disposable Sephadex A-25 column achieves a near qualitative separation of HETEpdG from γ-[^{32}P]ATP, and greatly reduces the handling problems of [^{32}P] as well as the efficiency of the final HPLC analysis.

Additional improvements of the method will follow from the inclusion of an internal standard to monitor the loss of HETEG in the DNA workup. Suitable candidates for this standard are being sought among other 7-substituted deoxyguanylic acids. Finally, the use of P1 nuclease prior to the labeling step to deplete unmodified 3'-deoxynucleotides will enhance the labeling of HETEpdG and permit the practical use of higher specific activity γ-[^{32}P]ATP.

CONCLUSIONS

The ^{32}P-postlabeling technique has been applied successfully to the detection of HETEG, the major adduct produced in DNA by sulfur mustard. The amount of DNA required for analysis is only 0.3 μg, and the method can detect less than one modification in 10^6 nucleotides. Further modification will increase sensitivity and extend the range of adducts which can be detected.

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